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L13: Entry 19 of 20

File: USPT

Jul 22, 2003

DOCUMENT-IDENTIFIER: US 6596499 B2

TITLE: Membrane molecule indicator compositions and methods

Abstract Text (1):

The invention provides membrane molecule indicators, including polypeptides, encoding nucleic acid molecules and cells containing such polypeptides and nucleic acid molecules. The invention membrane molecule indicators are characterized in that fluorescence resonance energy transfer (FRET) between a donor fluorescent domain and an acceptor fluorescent domain indicates a property of the membrane molecule. Also provided are methods of using the invention membrane molecule indicators to determine a property of a membrane molecule, and to identify compounds that modulates a property of a membrane molecule.

Brief Summary Text (5):

An early response to agonist stimulation of many tyrosine kinase and G-protein coupled receptors is the activation of the enzyme phospholipase C, which cleaves the lipid phosphatidylinositol 4,5-bisphosphate (PIP2) to generate second messengers that increase cytosolic free Ca.sup.2+ concentration. Although Ca.sup.2+ indicators and methods have been described that allow monitoring of Ca.sup.2+ concentration in single living cells with high spatial and temporal resolution, Ca.sup.2+ fluxes, being more distal to receptor activation, may not as faithfully report receptor activation levels as changes in PIP2 levels.

Brief Summary Text (6):

In a recently developed method for detecting PIP2 dynamics in living cells, a pleckstrin homology (PH) domain tagged with a green fluorescent protein (GFP) has been used. Detection of PIP2 hydrolysis was by in vivo visualization, such as by confocal imaging and post acquisition image analysis, of translocation of the fluorescence from the membrane to the cytosol. However, this method suffers from several disadvantages. First, it is hard to obtain quantitative data using confocal microscopy, since even minor focal drift and changes in cell morphology that often occur after stimulation render quantitative measurements unreliable. Second, it is difficult to visualize translocation in very flat cells or in cellular subregions. Third, at fast imaging rates, confocal imaging requires high excitation intensities that can cause severe cell damage in minutes. Fourth, the imaging approach is not easily extended to cell populations. Therefore, there exists a need to develop improved methods for detecting PIP2 dynamics in cells, and particularly methods amenable to high-throughput screening.

Brief Summary Text (9):

The invention provides a phosphatidylinositol 4,5-bisphosphate (PIP2) indicator. The indicator contains: (a) a first polypeptide having: (i) a pleckstrin homology (PH) domain; and (ii) a donor fluorescent domain (b) a second polypeptide having: (i) a pleckstrin homology (PH) domain; and (ii) an acceptor fluorescent domain; wherein fluorescence resonance energy transfer (FRET) between the donor domain and the acceptor domain indicates PIP2 levels.

Brief Summary Text (10):

Also provided is a nucleic acid kit, the nucleic acid molecule components of which encode a PIP2 indicator, the indicator containing: (a) a first polypeptide having: (i) a PH domain; and (ii) a donor fluorescent domain (b) a second polypeptide having: (i) a PH domain; and (ii) an acceptor fluorescent domain; wherein fluorescence resonance

energy transfer (FRET) between the donor domain and the acceptor domain indicates PIP2 levels.

Brief Summary Text (11):

Further provided is a method of indicating PIP2 levels in a cell. The method includes the steps of: (a) providing a cell containing a PIP2 indicator; and (b) determining FRET between the donor fluorescent domain and the acceptor fluorescent domain, wherein FRET between the donor domain and the acceptor domain indicates PIP2 levels in the cell.

Brief Summary Text (12):

The invention also provides a method of identifying a compound that modulates PIP2 levels in a cell. The method includes the steps of: (a) contacting a cell containing a PIP2 indicator with one or more test compounds; and (b) determining FRET between the donor fluorescent domain and the acceptor fluorescent domain following the contacting, wherein increased or decreased FRET following contacting indicates that the test compound is a compound that modulates PIP2 levels in the cell.

Drawing Description Text (2):

FIGS. 1A-L shows four exemplary membrane molecule indicator compositions. Solid bar: membrane anchoring domain. Hatched and open boxes: fluorescent donor domain or fluorescent acceptor domain. Thick semi-circle: MMID. Thin semi-circle: linker. Solid circle: membrane molecule. Solid triangle: represents an altered property of membrane molecule. (A-D): FRET is high due to association between membrane molecule indicator domain (MMID) and membrane molecule at the membrane. (E-H): FRET is low due to dissociation between MMID and membrane molecule, as a result of an altered property of membrane molecule. (I-L): FRET is low due to altered localization of membrane molecule.

Drawing Description Text (10):

FIG. 9 shows an exemplary membrane molecule indicator. Oval: membrane molecule. Trapezoid: MMID. The donor and acceptor fluorescent domains are indicated. Top: FRET is high due to association between MMID and the membrane molecule at the membrane and proximity of the donor and acceptor. Bottom: FRET is low due to relocation of membrane molecule and resulting separation of the donor and acceptor.

Drawing Description Text (11):

FIG. 10 shows an exemplary membrane molecule indicator. Oval: membrane molecule. Trapezoid: MMID. The donor and acceptor fluorescent domains are indicated. Top: FRET is low due to association between MMID and the membrane molecule at the membrane and separation of the donor and acceptor. Bottom: FRET is high due to relocation of membrane molecule and resulting proximity of the donor and acceptor.

Detailed Description Text (3):

The membrane molecule indicator compositions of the invention are characterized by a membrane molecule indicator domain, a donor fluorescent domain and an acceptor fluorescent domain. The donor fluorescent domain and acceptor fluorescent domain exhibit a characteristic fluorescence resonance energy transfer (FRET) when the membrane molecule indicator domain is associated with a membrane molecule at a membrane. This characteristic FRET observed when the membrane molecule indicator domain and membrane molecule are associated at the membrane differs from FRET observed when the membrane molecule indicator domain dissociates from the membrane molecule, or when the membrane molecule is no longer localized to the membrane. Therefore, FRET between the donor and acceptor fluorescent domains serves as an indicator of association at the membrane between the membrane molecule indicator domain and the membrane molecule, and thus serves as an indicator of a property of the membrane molecule.

Detailed Description Text (7):

In one embodiment, the membrane molecule indicator compositions of the invention contain (or encode) a single polypeptide that contains a membrane molecule indicator domain, a membrane anchor, a donor fluorescent domain and an acceptor fluorescent domain (shown schematically in FIG. 1A).

Detailed Description Text (8):

In an alternative embodiment, the membrane molecule indicator compositions of the invention contain (or encode) two polypeptides, one containing a membrane molecule indicator domain, the other containing a membrane anchor domain, one of which further contains a donor fluorescent domain, the other of which further contains an acceptor fluorescent domain (shown schematically in FIG. 1B).

Detailed Description Text (9):

In another embodiment, the membrane molecule indicator compositions of the invention contain (or encode) a single polypeptide that contains two membrane molecule indicator domains, a donor fluorescent domain and an acceptor fluorescent domain (shown schematically in FIG. 1C).

Detailed Description Text (10):

In yet another embodiment, the membrane molecule indicator compositions of the invention contain (or encode) two polypeptides, each containing a membrane molecule indicator domain, one of which contains a donor fluorescent domain and the other of which contains an acceptor fluorescent domain (shown schematically in FIG. 1D).

Detailed Description Text (11):

In a further embodiment, the membrane molecule indicator compositions of the invention contain (or encode) one polypeptide, containing a central membrane molecule indicator domain, with a donor fluorescent domain and an acceptor fluorescent domain at the termini (shown schematically in FIGS. 9 and 10).

Detailed Description Text (12):

It will be appreciated by the skilled person that the membrane molecule indicators shown in FIGS. 1, 9 and 10 can be modified in a variety of ways, so long as the donor and fluorescent domains are operably positioned so as to exhibit a characteristic FRET when the membrane molecule indicator domain and membrane molecule are associated at the membrane, which differs from FRET observed when the membrane molecule indicator domain dissociates from the membrane molecule, or when the membrane molecule is no longer localized to the membrane.

Detailed Description Text (13):

For example, the relative locations of the donor fluorescent domain and acceptor fluorescent domain with respect to a membrane anchoring domain can be reversed in the compositions shown in FIGS. 1A and B. The membrane molecule indicator compositions can also contain additional peptide or non-peptide domains, such as linker sequences between the donor fluorescent domain and acceptor fluorescent domain, or between a fluorescent domain and either the MMID or the membrane anchor. Likewise, either the donor or acceptor fluorescent domains shown in FIGS. 9 and 10 can optionally contain membrane anchor domains.

Detailed Description Text (24):

PIP2 resides at the plasma membrane of resting cells. Upon agonist stimulation of a receptor coupled to PLC, such as a tyrosine kinase receptor, or a G-protein coupled receptor (GPCR) that acts through a G.alpha.q-containing effector G protein, PIP2 is hydrolyzed to yield soluble IP3 and membrane bound DAG. PIP2 is then resynthesized and returns to the membrane. Accordingly, the abundance of PIP2 at the plasma membrane reports the activation state of a PLC-coupled receptor, in that high abundance of PIP2 at the plasma membrane indicates the resting state, and low abundance indicates agonistic activity through the receptor.

Detailed Description Text (25):

In an alternative embodiment, a membrane molecule is a membrane protein. Exemplary membrane proteins include integral membrane proteins such as cell surface receptors (e.g. G-protein coupled receptors (GPCRs), tyrosine kinase receptors, integrins and the like) and ion channels; and proteins that shuttle between the membrane and cytosol in response to signaling (e.g. Ras, Rac, Raf, G.alpha. subunits, arresting, Src and other effector proteins). In certain embodiments, when specifically indicated, excluded from the scope of the invention is a membrane molecule that is a GPCR.

Detailed Description Text (28):

In certain embodiments, such as when the membrane molecule indicator is designed to indicate activation state of a GPCR, the MMID can comprise a G-protein subunit, such

as a G.alpha., G.beta. or G.gamma. subunit. For example, high FRET between a G.alpha. subunit linked to a donor fluorescent domain and a G.beta. and/or G.gamma. subunit linked to an acceptor fluorescent domain (or vice versa) can indicate the inactive state of the GPCR, in which the trimeric G-protein complex is present at the membrane. In contrast, low FRET can indicate activation of the GPCR and dissociation of the G-protein complex. In other embodiments, when specifically indicated, excluded from the scope of the invention is an MMID which comprises a G-protein subunit.

Detailed Description Text (39):

In another embodiment, the phospholipid indicator domain is a lipid binding domain of a clathrin adaptor protein, such as residues 5-80 of AP-2 (.alpha.-subunit), which specifically associates with PtdIns (3, 4, 5)P.sub.3, or residues 1-304 of AP-3, which specifically associates with pyrophosphate (PP)-InsP.sub.5 (reviewed in Bottomley et al., supra (1998)). The sequences of a variety of lipid binding domains of clathrin adaptor proteins are known in the art.

Detailed Description Text (48):

Optionally, the membrane anchoring domain can be a second membrane molecule indicator domain that associates with a different membrane molecule than the first membrane molecule indicator domain, and that is not co-regulated with the first membrane molecule. For example, in order to determine membrane abundance of PIP2, an appropriate indicator composition can include a membrane molecule indicator domain that associates with PIP2 (e.g. a PH domain) fused to a donor fluorescent domain, and a membrane molecule indicator domain that associates with a different membrane molecule that is not co-regulated with PIP2 fused to an acceptor domain, which thus serves to anchor the acceptor domain to the plasma membrane.

Detailed Description Text (49):

As used herein, the terms "donor fluorescent domain" and "acceptor fluorescent domain" refer to a pair of moieties selected so as to exhibit fluorescence resonance energy transfer (FRET) when the donor moiety is excited with appropriate electromagnetic radiation or becomes luminescent.

Detailed Description Text (50):

The donor fluorescent domain is excited by light of appropriate intensity within its excitation spectrum, and emits the absorbed energy as fluorescent light. When the acceptor fluorescent domain is positioned to quench the donor fluorescent domain in the excited state, the fluorescence energy is transferred to the acceptor fluorescent domain, which can emit fluorescent light. FRET can be manifested as a reduction in the intensity of the fluorescent signal emitted from the donor fluorescent domain, by reduction in the lifetime of the excited state of the donor fluorescent domain, or by emission of fluorescent light at the longer wavelengths (lower energies) characteristic of the acceptor fluorescent domain. When the association between the MMID and the corresponding membrane molecule changes, the donor and acceptor fluorescent domains physically separate (or come closer together), and FRET is decreased (or increased) accordingly (see FIG. 1).

Detailed Description Text (51):

One factor to be considered in choosing the fluorescent domain pair is the efficiency of fluorescence resonance energy transfer between them. Preferably, the efficiency of FRET between the donor and acceptor moieties is at least 10%, more preferably at least 50% and even more preferably at least 80%. The efficiency of FRET can easily be empirically tested using the methods described herein and known in the art.

Detailed Description Text (52):

The efficiency and detectability of FRET also depend on the separation distance and the orientation of the donor and acceptor fluorescent domains, as well as the choice of fluorescent domains. Considerations for the choice of fluorescent domains are well known in the art, and described, for example, in U.S. Pat. Nos. 5,998,204 and 5,981,200. For example, it is preferred that the emission spectrum of the donor fluorescent domain overlap as much as possible with the excitation spectrum of the acceptor fluorescent domain. In addition, the excitation spectra of the donor and acceptor fluorescent domains should overlap as little as possible so that a wavelength region can be found at which the donor fluorescent domain can be excited selectively and efficiently without directly exciting the acceptor moiety. Likewise, the emission

spectra of the donor and acceptor fluorescent domains should have minimal overlap so that the two emissions can be distinguished. Furthermore, it is desirable that the quantum yield of the donor fluorescent domain, the extinction coefficient of the acceptor fluorescent domain, and the quantum yield of the acceptor fluorescent domain be as large as possible.

Detailed Description Text (53):

For example, in a suitable pair of fluorescent domains, the donor fluorescent domain is excited by ultraviolet light (<400 nm) and emits blue light (<500 nm), while the acceptor fluorescent domain is efficiently excited by blue light (but not by ultraviolet light) and emits green light (>500 nm). In an alternative pair of fluorescent domains, the donor fluorescent domain is excited by violet light (about 400-430 nm) and emits blue-green light (450-500 nm), while the acceptor fluorescent domain is efficiently excited by blue-green light (but not by violet light) and emits yellow-green light (about 520-530 nm).

Detailed Description Text (54):

Generally, the donor fluorescent domain and acceptor fluorescent domain will be fluorescent proteins, as described below. Alternatively, the donor can contain a tag, such as an artificial tetracysteine-based peptide tag, to which a cell permeable fluorescent label, such as FLASH-EDT.sub.2, can bind (e.g. Griffin et al., Science 281:269-272 (1998)).

Detailed Description Text (55):

Fluorescent proteins suitable for use as donor or acceptor fluorescent domains in the compositions and methods of the invention have been isolated from a number of species, including jellyfish (e.g. Aequorea species) and coral (e.g. Renilla species and Discosoma species).

Detailed Description Text (56):

In one embodiment, the donor and/or acceptor fluorescent domain is a "green fluorescent protein" or "GFP," such as a native GFP from an Aequorea or Renilla species, an ortholog of a GFP from another genus, or a variant of a native GFP with optimized properties. As used herein, the term "GFP variant" is intended to refer to polypeptides with at least about 70%, more preferably at least 75% identity, including at least 80%, 90%, 95% or greater identity to a native GFP, such as Aequorea victoria GFP.

Detailed Description Text (58):

GFP variants with optimized dimerization properties can also be prepared. It is postulated that the weak dimerization observed between GFPs (e.g. kD about 100 .mu.M) allows donor and acceptor fluorescent domains present on separate polypeptide chains (e.g. FIG. 1B or 1D) to associate at the membrane and exhibit FRET, even at low expression levels where based simply on polypeptide concentration at the membrane, FRET would not be expected. The dimerization is suitably weak so that once dissociated from the membrane or from the membrane molecule, the donor and acceptor fluorescent domains separate so as to no longer exhibit FRET. GFP variants with altered dimerization properties can be selected so as to optimize the differential in FRET between alternatives configurations. For example, GFP variants with slightly higher, but still moderate, dimerization (e.g. kD about 25 .mu.M) are expected to provide for suitably high FRET at the membrane even at low polypeptide expression levels, while still separating once dissociated from the membrane or from the membrane molecule.

Detailed Description Text (59):

Cyan fluorescent proteins (CFPs) are variant GFPs that contain the mutation Y66W with respect to Aequorea Victoria GFP. Yellow fluorescent proteins (YFPs) are variant GFPs that contain aromatic residues at position 203. Blue fluorescent proteins (BFPs) are variant GFPs that contain a Y66H mutation. A group of GFPs which lack the near-UV excitation peak, but retain the wild-type GFP emission peak, have Ser65 substitutions. Other variants of native GFPs with useful fluorescent properties are known in the art, or can be readily prepared by random or directed mutagenesis of a native GFP. Exemplary pairs of donor and acceptor fluorescent domains include BFP-GFP and CFP-YFP.

Detailed Description Text (60):

In another embodiment, the donor and/or acceptor fluorescent domain is a "DsRed," such as a native DsRed from a *Discosoma* species, an ortholog of DsRed from another genus, or a variant of a native DsRed with optimized properties (e.g. a K83M variant or DsRed2 (available from Clontech)). As used herein, the term "DsRed variant" is intended to refer to polypeptides with at least about 70%, more preferably at least 75% identity, including at least 80%, 90%, 95% or greater identity to a native DsRed, such as a *Discosoma* DsRed. Other variants of native DsReds with useful fluorescent properties are known in the art, or can be readily prepared by random or directed mutagenesis of a native DsRed (see, for example, Fradkov et al., FEBS Lett. 479:127-130 (2000)).

Detailed Description Text (61):

Other exemplary pairs of donor and acceptor fluorescent domains, respectively, include GFP-dsRED2 and YFP-dsRED2.

Detailed Description Text (62):

Included within the term "donor fluorescent domain" is a bioluminescent domain, such as luciferase from *Renilla*, related species, and variants thereof. *Renilla* luciferase emits blue light in the presence of an appropriate substrate, such as coelenterazin, which can be transferred to an appropriate fluorescent acceptor domain, such as a GFP, in a process called Bioluminescence Resonance Energy Transfer, or BRET. BRET is described, for example, in Angers et al., Proc. Natl. Acad. Sci. USA 97:3684-3689 (2000); Xu et al., Proc. Natl. Acad. Sci. USA 96:151-156 (1999); and components are commercially available from BioSignal Packard (Montreal, Canada). Those skilled in the art can readily apply the compositions and methods described herein with respect to FRET, to compositions and methods involving BRET.

Detailed Description Text (63):

In constructs in which the donor fluorescent domain and the acceptor fluorescent domain are present on the same polypeptide, the fluorescent domains can optionally be separated by a flexible "linker sequence." An appropriate linker sequence allows the donor and acceptor fluorescent domain to be functionally coupled when the single MMID (FIG. 1A), or pair of MMIDs (FIG. 1B), are associated with a membrane molecule, such that FRET is high, and functionally uncoupled when the MMIDs are not associated with the membrane molecule, such that FRET is low (FIGS. 1D and E). In order to optimize the FRET effect, the average distance between the donor and acceptor fluorescent domains should become less than about 10 nm when the MMID is associated with the membrane molecule (e.g. from 1 nm to 10 nm).

Detailed Description Text (71):

It will be appreciated that a nucleic acid molecule encoding a polypeptide containing a MMID and a donor fluorescent domain, and a nucleic acid molecule encoding a polypeptide containing a MMID and an acceptor fluorescent domain (e.g. FIGS. 1B and 1D) can optionally be present on the same vector or under the control of the same promoter. Such constructs are advantageous, for example, in simplifying introducing the nucleic acid molecules into a cell and in ensuring 1:1 stoichiometry of the donor and acceptor in the pair. Alternatively, the nucleotide sequences encoding the two polypeptides can be present on separate vectors or under the control of different promoters.

Detailed Description Text (84):

The invention also provides a method of determining a property of a membrane molecule. The method is practiced by (a) providing a cell or lipid bilayer comprising a membrane molecule indicator; and (b) determining FRET between the donor fluorescent domain and the acceptor fluorescent domain, wherein FRET between the donor domain and the acceptor domain is indicative of a property of the membrane molecule.

Detailed Description Text (85):

Also provided is a method of identifying a compound that modulates a property of a membrane molecule. The method is practiced by (a) contacting a cell or lipid bilayer comprising a membrane molecule indicator with one or more test compounds, wherein the cell or bilayer further comprises the membrane molecule; and (b) determining FRET between the donor fluorescent domain and the acceptor fluorescent domain following contacting, wherein increased or decreased FRET following contacting indicates that the test compound is a compound that modulates a property of the membrane molecule.

Detailed Description Text (89):

As an example, the methods of the invention can be used to identify test compounds that are agonists, antagonists, inverse agonists or natural ligands of receptors, including G-protein coupled receptors (described further below), tyrosine kinase receptors (e.g. PDGF, IGF, FGF and EGF receptors and the like) and integrins. In the methods of the invention, the basal level of FRET can be determined in an unstimulated lipid bilayer. The lipid bilayers can then be contacted with a test compound, and FRET compared with an unstimulated bilayer. FRET is advantageous over fluorescent visualization methods in that both increases and decreases, relative to the basal level, can be readily determined. Increased or decreased FRET relative to the basal level is a reflection of the activity of the test compound as an agonist, antagonist or inverse agonist of the signaling pathway linked to the membrane molecule.

Detailed Description Text (99):

As an example, the methods of the invention can be used to screen for G-protein coupled receptor (GPCR) agonists, antagonists and inverse agonists, as well as to identify the natural ligands of orphan GPCRs.

Detailed Description Text (100):

GPCRs are seven-transmembrane-domain polypeptides that transduce G-protein coupled signals in response to ligands. The natural agonists of different GPCRs range from peptide and non-peptide neurotransmitters, hormones and growth factors, to lipids, nucleoside-sugars, amino acids, light and odorants. GPCRs are involved in a variety of critical biological functions, including cell proliferation, differentiation and apoptosis. GPCRs have proven to be important targets of pharmaceuticals that affect a variety of diseases, including neurological and psychiatric disorders, vascular diseases, endocrinological disorders, and cancer. It is estimated that over 50% of current drugs are targeted towards GPCRs, and represent about a quarter of the 100 top-selling drugs worldwide.

Detailed Description Text (102):

As used herein, the term "G-protein" refers to a class of heterotrimeric GTP binding proteins, with subunits designated G.alpha., G.beta. and G.gamma., that couple to seven-transmembrane cell surface receptors to couple extracellular stimuli to intracellular messenger molecules. G-proteins are distinguished by their G.alpha. subunits. The more than (20) different G.alpha. subunits, encoded by (17) different genes, can be grouped into four major families: G.alpha.s, G.alpha.i, G.alpha.q, and G.alpha.12. Signaling through GPCRs that couple to G.alpha.q-containing G proteins activates PLC enzymes to hydrolyze PIP2 in the plasma membrane to DAG and IP3.

Detailed Description Text (104):

For example, in instances in which the membrane molecule indicator polypeptides are designed to indicate abundance of PIP2, cells (or other lipid bilayers) can contain a GPCR of interest, and optionally a G.alpha.q or G.alpha.16 (or chimeric or variant G.alpha. which functions as a G.alpha.q). The basal level of FRET between acceptor and donor fluorescent domains linked to a MMID (or two MMIDs) that associate with PIP2 can be determined. In response to agonist-induced signal transduction through the GPCR, PIP2 is hydrolyzed and FRET is decreased, as exemplified in the cells described in the Example, below. Likewise, antagonistic or inverse agonistic effects can be determined by an increase in agonist-induced, or basal, levels of FRET.

Detailed Description Text (105):

In the cells described in the Example, below, in which the MMID associates with PIP2 in the plasma membrane, FRET is high in unstimulated cells. In the presence of a test compound that activates PLC (e.g. bradykinin), FRET is significantly lower than in unstimulated cells, as PIP2 in the membrane is hydrolyzed, and the donor and acceptor fluorescent domains are no longer in close proximity. Thus, the compositions and methods described in the Example, below, can be used to identify and compare test compounds that stimulate the activation of PLC, that decrease the basal level of PLC activation, or that antagonize agonist-induced PLC activation.

Detailed Description Text (109):

Methods of determining and quantitating FRET at the single cell level, or in cell populations, are well known in the art or can be determined by the skilled person. For



example, FRET can be measured using dual emission fluorescence microscopy, as described in the Example, below. Alternatively, FRET can be measured using fluorescent microscopy imaging methodology, which allows for simultaneous recordings from multiple cells.

Detailed Description Text (110):

As a further example, FRET can be determined with fluorescent lifetime. Briefly, upon excitation with an ultrashort pulse of light (e.g. about 0.01 ns), fluorophores have a characteristic decay in emission that is single exponential, and may last 0.1-10 ns, dependent on the fluorophore and conditions. It has been shown that the presence of a FRET acceptor dramatically shortens the decay time of the donor, which can be detected either using direct monitoring of the decay time (time domain monitoring), or using sine-modulated light, in the frequency domain (see, for example, Verveer et al., Biophys. J., 78:2127-37 (2000)).

Detailed Description Text (116):

This example shows the preparation of two pairs of nucleic acid molecules of the invention. In the first pair, the first nucleic acid molecule encodes a polypeptide containing a membrane molecule indicator domain (PH domain) and a donor fluorescent domain (CFP), and the second nucleic acid molecule encodes a polypeptide containing a membrane molecule indicator domain (PH domain) and an acceptor fluorescent domain (YFP). In the second pair, the first nucleic acid molecule encodes a polypeptide containing a membrane molecule indicator domain (PH domain) and a donor fluorescent domain (CFP), and the second nucleic acid molecule encodes a polypeptide containing a membrane anchoring domain (CaaX) and an acceptor fluorescent domain (YFP).

Detailed Description Text (117):

This example also shows the use of the pairs of nucleic acid molecules to determine the abundance of a membrane molecule (PIP2), by determining FRET between the donor and acceptor fluorescent domains. High FRET results from high PIP2 abundance at the plasma membrane, which indicates the resting state of the cell; decreased FRET results from PIP2 hydrolysis, which indicates signaling through a G-protein coupled receptor linked to PLC activation.

Detailed Description Text (139):

For quantitation of expression levels of CFP-PH and YFP-PH, cellular fluorescence was compared to the fluorescence of a solution of known concentration of purified, bacterially expressed CFP-PH or YFP-PH, following the method of Miyawaki et al., "Calcium signaling: a practical approach," Oxford University Press (in press). In short, CFP-PH and YFP-PH were expressed as GST-fusion proteins, and purified on glutathione sepharose beads. Protein concentration was measured by the BCA\* Protein Assay (Pierce, Ill., USA). The solution (4.8  $\mu\text{M}$ ) was then introduced in a linear wedge-shaped chamber (0-170  $\mu\text{M}$  thickness) that was placed on the microscope (using NA 0.7 objective), and the position of the chamber was adjusted to give a fluorescence readout that matched that of a single, CFP or YFP expressing cell. The estimate of the fluorescent protein concentration in the cell was obtained by comparing the local thickness of the wedge to that of an average cell (17  $\mu\text{M}$ ). Relative amounts of CFP-PH and YFP-PH expression in cells were always determined under conditions of full cytosolic localization of the constructs.

Detailed Description Text (149):

During agonist-induced translocation, several factors may affect the fluorescent properties of these PH domain chimeras as well as the transfer of fluorescent energy between them (Tsien, Annu. Rev. Biochem. 61:509-544 (1998)). For example, the move away from a compartment adjacent to the lipophilic membrane could alter fluorescent characteristics, and is also likely to alter FRET by increasing the degree of rotational freedom. While the relative influence of increased rotational freedom on the translocation-induced decrease in FRET is difficult to assess in this model system, fluorescence changes were analyzed in some further detail.

Detailed Description Text (152):

To assess the effects of construct concentrations on FRET, cells expressing various levels of the chimeric proteins were compared. Intracellular fluorescent protein concentrations were estimated by comparing the emission intensities of individual cells to those of a solution of bacterially expressed, purified protein of known



concentration (Miyawaki et al., supra (in press); see Experimental Procedures). Based on these estimates, resonance could be observed in cells with expression levels between about 2-200  $\mu\text{M}$ , over a 100-fold concentration range. However, FRET was not observed in cells expressing less than about 1  $\mu\text{M}$  of each of the constructs. Very high expression levels, on the other hand, appeared to be detrimental to the cells (as judged from the appearance of membrane blebs and detachment of cells 2-3 days after transfection). Such cells were excluded from analysis. These data also revealed that PLC. $\delta$ .1PH-CFP expression levels (detected in fully translocated cells) did not differ more than about 2-fold from those of PLC. $\delta$ .1PH-YFP in most cells.

#### Detailed Description Text (153):

It was of interest to determine whether estimates of CFP and YFP concentrations can be used to calculate lipid concentrations and molecular proximity in the cells studied. Assuming a typical attached N1E-115 cell to be a pyramid having a 20  $\times$  20  $\mu\text{m}$  base and 10  $\mu\text{m}$  height (having 1.3 pL volume and 1100  $\mu\text{m}^2$  surface), and assuming that (I) the concentration of both chimera is 20  $\mu\text{M}$ ; (II) 50% of fluorophores are located at the membrane (complete translocation roughly doubles the fluorescence in the cytosol); (III) the distribution of fluorophores is homogenous along the membrane; and (IV) fluorophores are insensitive to the local environment, then the calculated mean distance between fluorophores is 7-8 nm, which is close to the reported Forster radius (50 Angstrom) for FRET between this pair of fluorophores (Tsien, supra (1998)). However, it should be emphasized that these assumptions are valid only as first approximations. For example, we and others (Tall et al., Curr. Biol. 10:743-746 (2000)) noted that GFP-PH is not homogeneously localized along the plasma membrane. Also, as discussed above, the spectral properties of the fluorescent proteins are sensitive to the microenvironment. Nevertheless, these data set a lower limit for the density of PIP2 molecules available for PH binding at the inner surface of the plasma membrane.

#### Detailed Description Text (159):

In very flat and small cell structures such as neurites and lamellipodia (below approximately 2  $\mu\text{m}$  in thickness), confocal imaging cannot detect translocation due to its inherent limit in z-axis resolution. However, in such cases changes in FRET can still be reliably detected as shown by the agonist-induced PLC activation recorded over a single neurite (FIG. 5B). FRET can also be recorded from cell populations (FIG. 5C) providing with an average response that would need analysis of hundreds of single cell recordings. Thus, detecting resonance between fluorescent protein-labeled PH domains overcomes a number of the limitations that are associated with confocal detection.

#### Detailed Description Text (161):

While PLC. $\delta$ .1PH-GFP has been introduced as an indicator of membrane PI(4, 5)P2 (Stauffer et al., Curr. Biol. 8:343-346 (1998); Varnai et al., supra (1998)), it also displays high affinity to IP3 (Hirose et al., Science 284:1527-1530 (1999)) which may exceed its affinity to PI(4, 5)P2, although it is difficult to accurately measure the latter as it is displayed in vivo. Based on such relative affinity estimates, Hirose and coworkers recently suggested that PLC. $\delta$ .1PH-GFP actually monitors IP3 increases rather than the changes in lipid levels in MDCK cells (Hirose et al., supra (1999)). They reported that microinjection of IP3 in MDCK cells was sufficient to cause displacement of PLC. $\delta$ .1PH-GFP from the membrane to the cytosol through competition for binding of the fluorescent construct to membrane PI(4, 5)P2. They also showed that expression of an IP3-5-phosphatase completely blocked the agonist-induced translocation of the fluorescent protein, and concluded that PI(4, 5)P2 changes do not make a significant contribution to the translocation response during stimulation.

#### Detailed Description Text (170):

These results thus suggest that PLC activation as assessed by FRET is a more faithful index of receptor activity than the more distal Ca $^{2+}$  transients. However, inactivation could occur at various steps in the signal cascade, including at the levels of receptor, G protein and PLC and, conceivably, also by modulation (upregulation) of PI(4, 5)P2 resynthesis. To test whether there is desensitization at the level of PLC, G proteins were directly activated using A1F $^{sup.4-}$  (FIG. 8A). While onset of A1F $^{sup.4-}$  induced PLC activation was slow, no desensitization was observed in any of these experiments. Similarly, cells expressing a constitutively active G. $\alpha$ .q mutant showed mostly cytosolic localization of PLC. $\delta$ .1 PH-GFP domains

for at least 2 days (FIG. 8B). Control transfection with activated G.alpha.12 had no effect. At lower expression levels, the activating mutant G.alpha.q induced sustained partial translocation that also persisted for several days. These experiments suggested that no significant desensitization occurs downstream of Gq and PLC. In line with this notion, significant heterologous desensitization between sequentially added agonists was not observed (compare e.g. FIGS. 5 and 7, last panel), whereas prolonged exposure of cells to each individual agonist induced complete (homologous) desensitization.

Detailed Description Text (171):

To further determine whether such monitoring of PLC activity truly follows receptor activity (in other words coupling and uncoupling between receptors and G proteins), the FRET responses of N1E-115 cells expressing either the wild-type NK2 receptors or a C-terminally truncated form, which is greatly impaired in its ability to desensitize (Alblas et al., J. Biol. Chem. 270: 8944-8951 (1995)), were compared. After stimulation of the wild-type human NK2 receptors the translocation response decays towards baseline within minutes (average 50% recovery time 83.+-.38 s, n=25; compare FIGS. 7 and 8C). Application of short pulses of agonist using a puffer pipette resulted in incomplete desensitization, and decayed significantly faster (45.+-.7 s, n=60, FIG. 8C) between applications of stimuli due to the rapid dissociation of the ligand from the receptor (Vollmer et al., J. Biol. Chem. 274:37915-37922 (1999)). Conversely, stimulation of a C-terminally truncated mutant human NK2 receptor, that was reported to be transforming in Rat-1 fibroblasts, and which has been found to display prolonged coupling to PLC (Alblas et al., supra (1995); Alblas et al., EMBO J. 15:3351-3360 (1996); Alblas et al., J. Biol. Chem. 268:22235-22238 (1993)) induced a much prolonged cytosolic translocation as assessed in FRET analysis (FIG. 8C). However, in the majority of cells, the FRET signal eventually slowly returned to baseline (FIG. 8D; note the different time scale), with an average 50% recovery time of 1365.+-.599 s (n=19) in the truncated receptor. This result indicates the existence of an alternative and much slower desensitization mechanism that functions even in NK2 receptors lacking the C-terminus. The kinetics of this slow desensitization closely paralleled those of receptor internalization (not shown), suggesting that one of the main determinants for termination of NKA-induced PLC signaling could be receptor internalization. Analysis of receptor activity by monitoring PLC activity by FRET will greatly aid further studies addressing these questions in more detail.

Detailed Description Text (173):

Analysis of the translocation responses suggests that localization of PLC.delta.1PH-GFP largely reports PI(4, 5)P2 dynamics, although at high concentrations IP3 can also contribute to translocation of the PH domains to the cytosol. Comparison of the Ca.sup.2+ and FRET-recorded responses of several agonists of GPCRs suggest that PLC activation detected by FRET is a more faithful reflection of receptor activity than the Ca.sup.2+ signal and that little if any "desensitization" or "uncoupling" occurs beyond the levels of G proteins.

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Janetopoulos et al., "Receptor-mediated activation of Heterotrimeric G-proteins in Living Cells," Science 291:2408-2411 (2001).

Other Reference Publication (20):

Komatsuzaki et al., "A Novel System that Reports the G-proteins Linked to a Given Receptor: A Study of Type 3 Somatostatin Receptor," FEBS Lett., 406:165-170 (1997).

Other Reference Publication (23):

Miyawaki et al., "Fluorescent Indicators for Ca<sup>sup.2+</sup> Based on Green Fluorescent Proteins and Calmodulin," Lett. Nat., 388:882-887 (1997).

Other Reference Publication (38):

Tsien, "The Green Fluorescent Protein," Annu. Rev. Biochem., 67:509-544 (1998).

Other Reference Publication (48):

Zhang et al., "Inhibition by Toxin B of Inositol Phosphate Formation Induced by G Protein-coupled and Tyrosine Kinase Receptors in N1E-115 Neuroblastoma Cells: Involvement of Rho Proteins," Mol. Pharmacol., 50:864-869 (1996).

Other Reference Publication (50):

Bastiaens and Jovins, "Microspectroscopic imaging tracks the intracellular processing of a signal transduction protein: Fluorescent-labeled protein kinase C .beta.I," Proc. Natl. Acad. Sci. USA 93:8407-8412 (1996).

## CLAIMS:

1. A phosphatidylinositol 4,5-bisphosphate (PIP2) indicator, said indicator comprising: (a) a first polypeptide comprising: (i) a pleckstrin homology (PH) domain; and (ii) a donor fluorescent domain (b) a second polypeptide comprising: (i) a pleckstrin homology (PH) domain; and (ii) an acceptor fluorescent domain; wherein fluorescence resonance energy transfer (FRET) between said donor domain and said acceptor domain indicates PIP2 levels.
3. The indicator of claim 1, wherein said donor fluorescent domain is selected from the group consisting of a GFP and a dsRED.
4. The indicator of claim 1, wherein said donor fluorescent domain is a CFP.
5. The indicator of claim 1, wherein said acceptor fluorescent domain is selected from the group consisting of a GFP and a dsRED.
6. The indicator of claim 1, wherein said acceptor fluorescent domain is a YFP.
8. A nucleic acid kit, the nucleic acid molecule components of which encode a PIP2 indicator, said indicator comprising: (a) a first polypeptide comprising: (i) a PH domain; and (ii) a donor fluorescent domain (b) a second polypeptide comprising: (i) a PH domain; and (ii) an acceptor fluorescent domain; wherein fluorescence resonance energy transfer (FRET) between said donor domain and said acceptor domain indicates PIP2 levels.
10. The kit of claim 8, wherein said donor fluorescent domain is selected from the group consisting of a GFP and a dsRED.
11. The kit of claim 8, wherein said donor fluorescent domain is a CFP.
12. The kit of claim 8, wherein said acceptor fluorescent domain is selected from the group consisting of a GFP and a dsRED.
13. The kit of claim 8, wherein said acceptor fluorescent domain is a YFP.
15. A method of indicating PIP2 levels in a cell, comprising: (a) providing a cell containing the PIP2 indicator of claim 1; and (b) determining FRET between said donor fluorescent domain and said acceptor fluorescent domain, wherein FRET between said donor domain and said acceptor domain indicates PIP2 levels in the cell.
17. The method of claim 15, wherein said donor fluorescent domain is selected from the group consisting of a GFP and a dsRED.
18. The method of claim 15, wherein said donor fluorescent domain is a CFP.
19. The method of claim 15, wherein said acceptor fluorescent domain is selected from the group consisting of a GFP and a dsRED.

20. The method of claim 15, wherein said acceptor fluorescent domain is a YFP.
21. The method of claim 15, wherein said cell recombinantly expresses a G-protein coupled receptor.
22. A method of identifying a compound that modulates PIP2 levels in a cell, comprising: (a) contacting a cell containing the PIP2 indicator of claim 1 with one or more test compounds; and (b) determining FRET between said donor fluorescent domain and said acceptor fluorescent domain following said contacting, wherein increased or decreased FRET following said contacting indicates that said test compound is a compound that modulates PIP2 levels in the cell.
24. The method of claim 22, wherein said donor fluorescent domain is selected from the group consisting of a GFP and a dsRED.
25. The method of claim 22, wherein said donor fluorescent domain is a CFP.
26. The method of claim 22, wherein said acceptor fluorescent domain is selected from the group consisting of a GFP and a dsRED.
27. The method of claim 22, wherein said acceptor fluorescent domain is a YFP.
30. The method of claim 22, wherein said cell recombinantly expresses a G-protein coupled receptor.